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Advances in Brief

Down-Regulation of Regulatory Subunit Type 1A of Protein Kinase A Leads to Endocrine and Other Tumors

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Abstract

Mutations of the human type Iα regulatory subunit (RIα) of cyclic AMP-dependent protein kinase (PKA; *PRKAR1A*) lead to altered kinase activity, primary pigmented nodular adrenocortical disease, and tumors of the thyroid and other tissues. To bypass the early embryonic lethality of *Prkar1a*$^{-/-}$ mice, we established transgenic mice carrying an antisense transgene for *Prkar1a* exon 2 (X2AS) under the control of a tetracycline-responsive promoter. Down-regulation of *Prkar1a* by up to 70% was achieved in transgenic mouse tissues and embryonic fibroblasts, with concomitant changes in kinase activity and increased cell proliferation, respectively. Mice developed thyroid follicular hyperplasia and adenomas, adrenocortical hyperplasia, and other features reminiscent of primary pigmented nodular adrenocortical disease, histiocytic and epithelial hyperplasias, lymphomas, and other mesenchymal tumors. These were associated with allelic losses of the mouse chromosome 11 *Prkar1a* locus, an increase in total type II PKA activity, and higher RIIβ protein levels. This mouse provides a novel, useful tool for the investigation of cyclic AMP, RIα, and PKA functions and confirms the critical role of *Prkar1a* in tumorigenesis in endocrine and other tissues.

Introduction

PRKAR1A-inactivating mutations cause primary pigmented nodular adrenocortical disease, Carney complex, a multiple neoplasia syndrome (1–3), and sporadic endocrine tumors (4–6). PKA is a serine-threonine kinase that mediates cyclic AMP (cAMP) regulation for a variety of cellular processes, including DNA replication and cell proliferation (7, 8). There are four PKA regulatory subunits [*PRKAR1A* (RIα), *PRKAR1B* (RIβ), *PRKAR2A* (RIIα), and *PRKAR2B* (RIIβ)] and three catalytic subunits [*PRKACA* (Cα), *PRKACB* (Cβ), and *PRKARCG* (Cγ; ref. 9)]; there are two isoforms of the PKA holoenzyme: type I and type II; they contain homodimers of either the RIα and RIβ or the RIIα and RIIβ subunits, respectively (8, 9). Type I PKA is the physiologic mediator of cAMP actions; in basal states, the catalytic subunits bind preferentially to type II regulatory subunits, whereas binding to type I subunits is favored in stimulated states (8, 10). Studies, mostly in cancer cell lines, have shown RIα overexpression, rather than underexpression (8, 11). However, mice with increased levels of RIα protein, such as the *Prkar1b*$^{-/-}$, *Prkar2a*$^{-/-}$, and *Prkar2b*$^{-/-}$ mice, have not shown an increased frequency of tumors (9, 12, 13). *In vivo* investigations of RIα function have been hindered by the early embryonic lethality demonstrated by the homozygous *Prkar1a*$^{-/-}$ mice (14). While young, heterozygous *Prkar1a*$^{+/-}$ mice of a mixed genetic background did not have an abnormal phenotype (9, 14), there have been recent, preliminary reports that mice with inactivated *Prkar1a* develop mesenchymal tumors at an older age (15, 16). In the present study, we tested the hypothesis that *Prkar1a* reduction by 50% or more is necessary for induction of tumors in mice; to avoid potential early lethality, we designed a construct that would allow us to delay the onset of gene down-regulation until after birth: We created a transgenic (*Tg*) mouse carrying an antisense sequence for exon 2 of the *Prkar1a* gene (X2AS-RIα) under the control of a tetracycline-responsive promoter; then the *Tg(Prkar1a*x2as)1Stra* mice were crossed with those expressing tetracycline transactivator (tTA), to produce the *Tg(Prkar1a*x2as)1Stra, Tg(tTAhCMV)3Uh* lines (the tTA/X2AS mice) that we used as a model of *Prkar1a* down-regulation.

Materials and Methods

Construction of the Transgene and Generation of Transgenic Mice. The Animal Care and Use Committee of the National Institute for Child Health & Human Development approved protocol ASP 01-003 for our animal studies. The mouse *Prkar1a* exon 2 (X2) sequence was amplified by PCR from normal mouse genomic DNA using the following primers: X2-*Prkar1a*-S (sense), 5′-GAACATGGC GTCTGGCAGT-3′, and X2-*Prkar1a*-AS (antisense), 5′-AAGGAATGCCATGGGCCTCT-3′; the amplicon was then cloned into the pCR2.1 vector (TOPO-TA cloning; Invitrogen, Carlsbad, CA). After verification of the proper sequence, the fragment was subcloned as an *Xba*I-*Spe*I fragment into the *Xba*I site of pTRE2 used in the tetracycline (Tet)-on or -off regulatory systems (17) (Clontech-BD Biosciences, Palo Alto, CA; Fig. 1*A*). After excision with *Aat*II and *Ase*I, the 2016-bp linear construct was gel purified (Qiagen, Inc., Valencia, CA), EtOH precipitated, and resuspended in 10 mmol/L Tris (pH 8.0) and 0.25 mmol/L EDTA buffer at a concentration of 35 ng/μL. Microinjection into C57BL/6 × SJL hybrid mouse eggs and their transfer into pseudopregnant foster mothers were completed at DNX Transgenic Sciences (Cranbury, NJ). Integration-positive mice, the *Tg(Prkar1a*x2as)1Stra* mice, were identified by PCR genotyping. Three founder lines were established that were maintained independently. One line had poor reproductive efficiency; the other two were used for subsequent studies. Subsequent confirmation of the presence of the X2AS construct in these mice was by PCR amplification DNA extracted from mouse tails (Qiagen, Inc.) using the following primers: X2AS-#5-L (sense), 5′-GTACCCGGGGATCCTCTAGT-3′, and X2AS-#5-R (antisense), 5′-GGTACCAAGTT CCCGCTTAA-3′, which produces an amplicon of 274 bp (Fig. 1*B*). To generate mice in which the X2AS-R1α construct would be regulated according to the *Tet-off* system (17), the *Tg(Prkar1a*x2as)1Stra* mice were crossed with mice expressing the tTA (the *Tg(tTAhCMV)3Uh* mice; JAX Research Systems, Bar Harbor, ME], to produce the *Tg(Prkar1a*x2as)1Stra*, *Tg(tTAhCMV)3Uh* line (the tTA/X2AS mice). These mice were born normally and at the expected Mendelian frequency. Genotypes were determined by PCR amplification of mouse genomic tail DNA using the X2AS-R1α primers described above and the following tTA

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Note: Supplementary data for this article can be found at Cancer Research Online (http://cancerres.aacrjournals.org). K. Griffin and L. Kirschner contributed equally to this work. L. Kirschner is currently at the Department of Internal Medicine, Divisions of Human Cancer Genetics and Endocrinology, Ohio State University, Columbus, Ohio.

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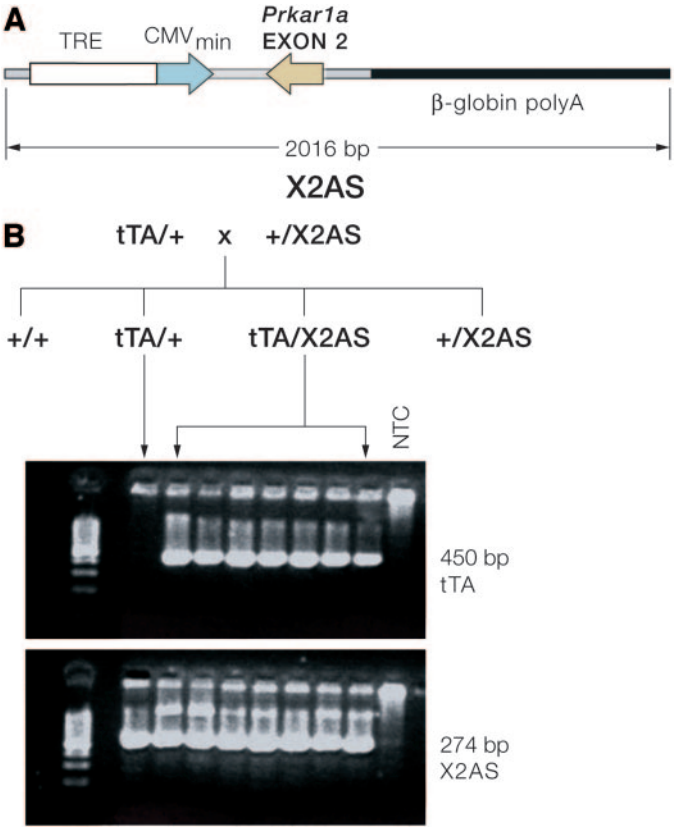

Fig. 1. Generation of the *Tg(Prkar1a*x2as)1Stra, Tg(tTAhCMV)3Uh* lines (the tTA/X2AS mice). *A*, schematic representation of the *Prkar1a*x2as* construct involving the antisense sequence of the exon 2 of the mouse *Prkar1a* gene cloned into the *Xba*I site of the pTRE2 plasmid used in the Tet-off regulatory system. *B*. The *Tg(Prkar1a*x2as)1Stra* line was crossed with the commercially available *Tg(tTAhCMV)3Uh* line expressing tTA to generate the double transgenic tTA/X2AS mouse line; the presence of the 450- and 274-bp amplicons from the *Prkar1a*x2as* and *tTAhCMV* constructs, respectively, was used for confirmation of the genotype in all experimental animals.

primers: tTA-L (sense), 5′-CGCTGTGGGGCATTTTACTTTAG-3′, and tTA-R (antisense), 5′-CATGTCCAGATCGAAATCGTC-3′, which produced an amplicon of 450 bp (Fig. 1*B*). Over the course of 14 generations, the tTA/X2AS mice were back-crossed to a mixed C57BL/6 background. Silencing the expression of the X2AS-R1α construct during pregnancy with the tetracycline congener doxycycline [by adding 200 μg/mL of the antibiotic to their drinking water, which was filter-sterilized and renewed every 48 to 72 hours (17)] did not affect the phenotype or number of tTA/X2AS newborn mice (data not shown).

Mouse Embryonic Fibroblasts and Mouse Phenotyping. Mouse embryonic fibroblasts were prepared using standard procedures from e12-14 embryos and maintained in Dulbecco's modified eagle medium supplemented with 15% fetal bovine serum, 1% penicillin-streptomycin, and 1% glutamine (mouse embryonic fibroblast medium; Life Technologies, Gaithersburg, MD). For all analyses, the tTA mice and the corresponding mouse embryonic fibroblast lines were considered the control group. All mice were fed and maintained similarly in an in-house animal facility; they were weighed weekly. For phenotyping, age- and gender-matched tTA and tTA/X2AS mice were sacrificed approximately every 2 months, from 4 to 18 months of age. Tissue fragments were snap-frozen and stored at −70°C until processing for PKA assay activity, mRNA, and protein studies. Most histopathologic analysis was done in our laboratory and at Molecular Histology, Inc., (Gaithersburg, MD) or at animal facilities at the NIH (Frederick Cancer Research and Development Center, National Cancer Institute, Frederick, MD and the Division of Veterinary Resources, NIH, Bethesda, MD).

***In situ* Hybridization with *Prkar1a*- and *X2AS*-Specific Probes and Messenger Ribonucleic Acid Studies.** Tissues were embedded in OCT and sectioned at −23°C. Ten-μm sections were thaw-mounted on poly-L-lysine–coated slides for histochemical analysis. The following probes were used: *Prkar1a*-X2-sense 1, 5′-gcacag-ctgcacgattggagtccttcagcagggcctggata-3′, and *Prkar1a*-X2-sense 2, 5′-gcttctgcacatag-agctcgcattcccggagactccgctc-3′ for sequences 667 to 706 and 711 to 750 of the *Prkar1a* gene exon 2, respectively. For detection of the antisense sequence covering the same regions of the *Prkar1a-X2AS* construct, the following primers were used: *Prkar1a*-X2AS-Anti-1, 5′-tatccaggccctgctgaaggactccatcgtgcagc-tgtgc-3′, and *Prkar1a*-X2AS-Anti-2, 5′-gagcggagtctccgggaatgcgagctctatgtgcagaagc-3′. Probes were 3′–end-labeled with [^{35}S]dATP and Terminal Transferase (Roche Diagnostics, Indianapolis, IN) and hybridized at 37°C overnight; after hybridization, sections were exposed to film and later dipped in Kodak NTB2 emulsion and exposed for 12 days at 4°C, following procedures described elsewhere (18). Serial sections were hybridized to sense probes and processed together with antisense probe-hybridized sections. For all analyses, background was subtracted from the hybridization signal and measured by investigators blinded to the origin of the specimen using NIH image software version 1.57, as described elsewhere (18). Quantitative real-time PCR was performed using the GeneAmp 5700 Sequence detection system and SDS software (Applied Biosystems, Foster City, CA) under standard conditions (40 cycles of 95°C for 15 seconds and 60°C for 60 seconds). All primer/probe sets were obtained from Applied Biosystems: *Prkar1a*, *Prkar2a*, *Prkar1b*, and *Prkarca*. The primer/probe set for *Prkar2b* was designed using Assay by Design (Applied Biosystems): MPRKAR2B-X3 × 4F, 5′-GGTCTGTGCAGAAGCTTATAATCCT-3′; MPRKAR2B-X3 × 4R, 5′-CCTCTTGCAATCTGTTTCTCTGATC-3′; and MPRKAR2B-X3 × 4M2, FAM-CAGAGTCCAGGATAATAC-3′. Rodent glyceraldehyde-3-phosphate dehydrogenase (reverse primer, forward primer, and probe) was used as a control.

Mouse Embryonic Fibroblast, Tissue Immunoblotting, and AMP-Dependent Protein Kinase Assays. Cell lysates were obtained when stimulated cells were placed on ice immediately after incubation and centrifuged at 10,000 × *g* for 20 seconds. Cell pellets were resuspended in lysis buffer (pH 7.3) containing 20 mmol/L HEPES, 10% glycerol, 1% Triton X-100, 50 mmol/L NaF, 1 mmol/L $NaVO_4$, and protease inhibitors (0.02 mg/mL aprotinin, 0.02 mg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, and 2.5 mmol/L 4-Npp), and homogenized at 4°C. Immunostaining was done with monoclonal antibodies for the PKA subunits as described previously (19) and specified by the manufacturer (BD Transduction Laboratories, San Jose, CA): RIα (610609), 1:1000; RIIα (612242), 1:1000; RIIβ (610625), 1:500; Cα (610980), 1:1000; the secondary antibody was an horseradish peroxidase–conjugated antimouse immunoglobulin (Ig) G (DC02L; Oncogene Science, Cambridge, MA), 1:1000. For mouse tissue analysis, polyclonal primary antibodies were used: goat anti-RIα (sc-18800) at 1:100 dilution; goat anti-RIIβ (sc-18804) at 1:100; rabbit anti-Cα (sc-903) at 1:20 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-RIIβ (539234) at 1:1000; and anti-RIβ (539233) at 1:1000 (Calbiochem, San Diego, CA). Horseradish peroxidase–conjugated secondary antibodies in these cases were either mouse antigoat IgG (Santa Cruz Biotechnology, sc-2354) at 1:1000 or mouse anti-rabbit IgG (DC03L; Oncogene Science) at 1:1000. Blots were developed with the ECL detection system (Amersham Biosciences Corp., Piscataway, NJ). Bands were detected by ECL reagent and quantitated by densitometer scanning (Molecular Dynamics, Sunnyvale, CA) and normalized against β-actin (ab8227; 1:25,000; Abcam, Cambridge, MA) detected on the same blot. PKA assays on mouse tissues were performed as described previously (1, 4, 5, 20).

Statistical Analyses. Age- and gender-matched tTA mice were the control group. Phenotypic differences between C57BL/6 mice, transgenic tTA, and transgenic tTA/X2AS mice were analyzed by χ^2 testing. The Kaplan-Meier analysis was used for comparing survival between the two mouse lines. Band densities from immunoblots, quantitative real-time PCR, proliferation, and PKA assay data from all tissues were compared with the STATISTICA software (StatSoft, Inc., Cary, NC) using the *t* test for individual comparisons between the two mouse lines; $P < 0.05$ was considered significant.

Results

The *Tg(Prkar1a*x2as)1Stra* mice, carrying the (X2AS-RIα) construct (Fig. 1*A*) were crossed with mice expressing the tTA, to produce the *Tg(Prkar1a*x2as)1Stra, Tg(tTAhCMV)3Uh* line (the tTA/X2AS mice) that we used as a model of *Prkar1a* down-regulation (Fig. 1*B*). Mouse embryonic fibroblasts from these mice showed regulation of the expression of the tTA-driven *Prkar1a-X2AS* con-

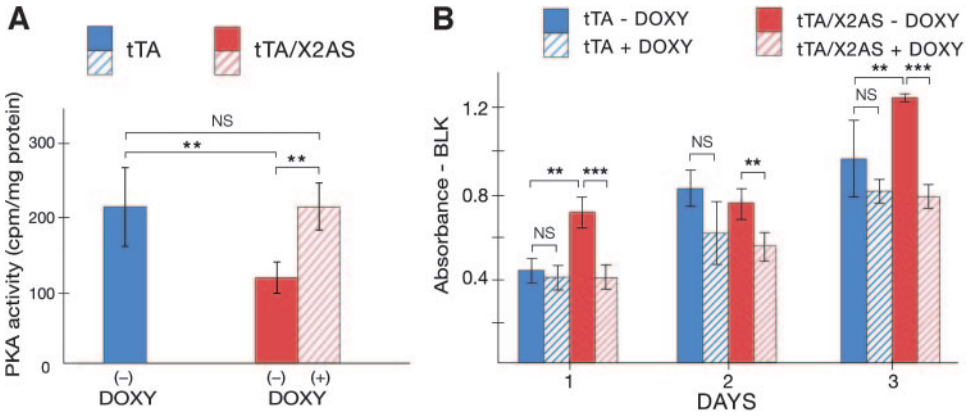

Fig. 2. PKA activity in control tTA (*blue*) and tTA/X2AS (*red*) mouse embryonic fibroblast cells. *A*. In the absence of doxycycline (*DOXY*), cAMP-stimulated PKA-specific activity decreased as the tTA/X2AS construct decreased *Prkar1a* levels. In the presence of DOXY, PKA activity returns to the control mouse embryonic fibroblast levels. *B*, DOXY had no effect on the proliferation rate of control mouse embryonic fibroblast cells over 3 days; DOXY decreased the enhanced proliferation rate of tTA/X2AS mouse embryonic fibroblasts down to the level of control cells.

struct by the tetracycline congener, doxycycline; significant PKA activity changes accompanied the *Prkar1a* message and protein modifications (Fig. 2*A*): Expression of the antisense construct led to a decrease in PKA-specific activity, whereas doxycycline restored enzymatic activity levels to those of the control cells. Growth of tTA/X2AS mouse embryonic fibroblasts over 3 days was greater than both control (only tTA-bearing) mouse embryonic fibroblasts and tTA/X2AS mouse embryonic fibroblasts treated with doxycycline ($P < 0.05$); doxycycline did not have a significant effect on the growth of the control cells ($P > 0.1$; Fig. 2*B*).

In tissues from adult tTA/X2AS mice of both genders, there was down-regulation of the *Prkar1a* message and protein levels by as much as 70 and 60%, respectively, compared with tissues from matched control tTA mice, but there was tissue-dependent variability. *In situ* hybridization of liver parenchyma using probes specific for the sense and antisense sequences (Fig. 3*A–H*) showed reduction of the *Prkar1a* mRNA by 69% (Fig. 3*I*). In adrenal tissue, the reduction of *Prkar1a* mRNA was 45% (Fig. 3*J*). RIα protein levels in tTA/X2AS mice decreased by 60 and 40% in liver and adrenal tissue, respectively; Western blot analysis from kidney and adrenal tissues were also consistent with an approximately 50% decrease of RIα protein levels in these mice compared with the same tissues from matched tTA controls (Supplementary Fig. 1).

In both mRNA and protein analyses, we found significant changes in the other PKA subunits. As shown in Fig. 3*J*, down-regulation of *Prkar1a* mRNA was associated with down-regulation of *Prkar1b*, *Prkar2a*, and *Prkaca* mRNAs in the adrenal gland; *Prkar2b* message levels remained unchanged. Likewise, RIIβ protein was increased in tissue lysates from the tTA/X2AS mice, whereas RIβ, RIIα, and Cα levels were all significantly decreased (Supplementary Fig. 1). As in mouse embryonic fibroblasts, tissues from the tTA/X2AS mice showed significant PKA activity changes: PKA assays from a variety of tissues showed the expected decrease in PKA-specific activity and increase in total kinase activity in response to cAMP (Supplementary Fig. 2)

Histologic abnormalities in the thyroid and adrenal glands, lymphoproliferative disease, and mesenchymal tumors developed in some mice as early as 4 to 6 months of age (Supplementary Table 1). Death

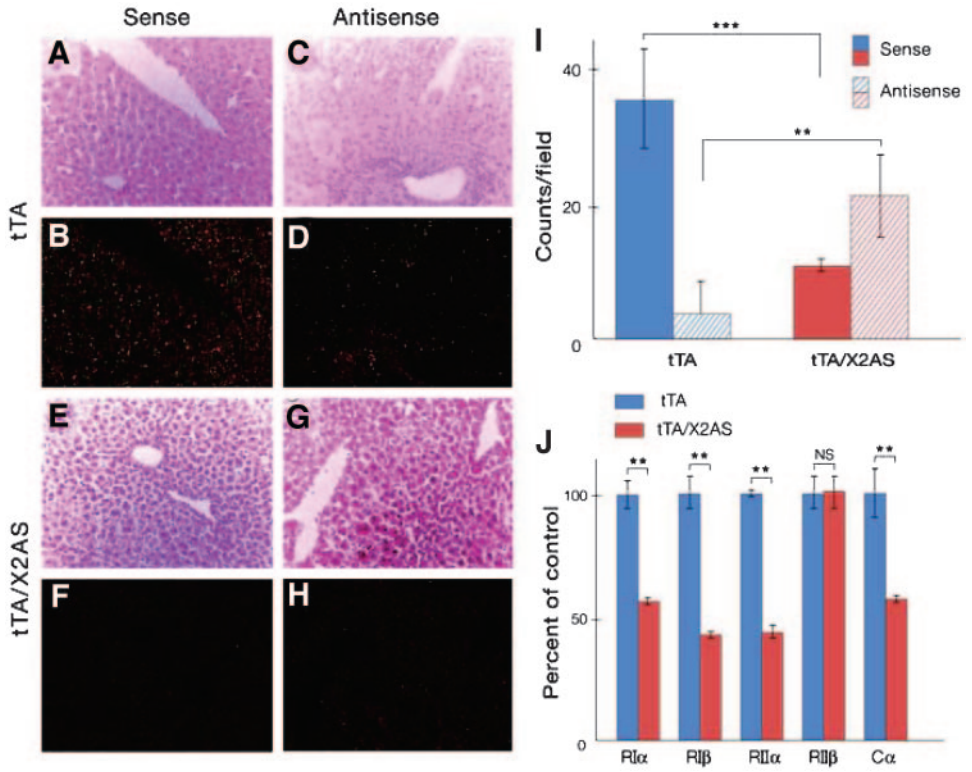

Fig. 3. Reduction of *Prkar1a* mRNA levels in tTA/X2AS mice. *A–H*. *In situ* hybridization of liver tissue from control tTA (*A* and *C*) and double transgenic tTA/X2AS mice (*E* and *G*) with antisense (*B* and *F*) and sense (*D* and *H*) *Prkar1a* probes shows reduction of the sense message (*B versus F*) and detection of the antisense sequence (*H*). *I*, statistical analysis of the signal reduction of *Prkar1a* mRNA and the expression of the antisense sequence in tTA/X2AS mice (*red*) *versus* tTA mice (*blue*) as detected by *in situ* hybridization. *J*, quantitative analysis of mRNA levels for the main mouse PKA subunits *Prkar1a*, *Prkar1b*, *Prkar2a*, *Prkar2b*, and *Prkaca* in adrenal tissue from the control tTA (*blue*) and double transgenic tTA/X2AS (*red*) mice. **, $P < 0.05$; ***, $P < 0.001$.

rate differences became significant after 16 months of age (Supplementary Fig. 3); the most frequent cause of death was a pulmonary or kidney condition related to a lymphoma, histiocytic sarcoma, another lympho- or histio-proliferative syndrome, or a mesenchymal tumor. Histiocytic hyperplasia, sarcomas, and lymphomas developed in tTA/X2AS mice both in primary lymphoid organs (thymus, spleen, lymph nodes, and Peyer's patches) and other tissues (liver and kidney; Fig. 4). Large, macroscopically visible, and occasionally metastatic tumors grew in tTA/X2AS mice (Supplementary Table 1). These lesions were of mesenchymal origin and corresponded to tumors seen in Carney complex patients (data not shown).

Discussion

Our study extends the findings of Amieux *et al.* (14) and Kirschner *et al.* (1) in RIα-deficient mice and patients with Carney complex, respectively. More recently, *Prkar1a*$^{+/-}$ mice were shown to develop tumors at a late age (16), but complete phenotyping of these animals is still lacking; tTA/X2AS mice grew tumors of mesenchymal origin at an apparently earlier age that what these preliminary reports indicate for *Prkar1a*$^{+/-}$ mice (16). Overall, there were significant similarities between the phenotype of tTA/X2AS mice and Carney complex patients, but also differences. First, there was a high incidence of thyroid lesions in the tTA/X2AS mice, which are extraordinarily rare in wild-type animals but frequent among Carney complex patients (1, 2). Second, the tTA/X2AS mice had adrenal lesions, some of them very similar to primary pigmented nodular adrenocortical disease (data not shown). Third, the tTA/X2AS mice developed mesenchymal and epithelial hyperplasias in a variety of tissues, including histiocytosis in multiple organs to glandular ectasia, as well as spindle cell schwannoma and squamous papilloma tumors. Carney complex patients also develop mesenchymal tumors, as typified by myxomas; these are the most frequent nonendocrine tumors in Carney complex. Fourth, the pattern of alterations of PKA activity in tissues from tTA/X2AS mice are overall similar to those seen in tumors from Carney complex patients or with spontaneous mutations in the *PRKAR1A* gene.

How does *Prkar1a* down-regulation cause tumors in endocrine and other tissues? It is clear that *Prkar1a*$^{-/-}$ and tTA/X2AS mouse embryonic fibroblasts maintain increased proliferation rates (14). This was associated with a switch to mostly type II PKA activity and an increase in RIIβ subunit. It has been suggested that type I PKA is associated with growth and proliferation, whereas type II PKA is associated with increased differentiation and decreased proliferation (8, 11). However, primary cultures of melanocytes and mammary cells (with mostly type II PKA) are stimulated by cAMP, whereas the mouse Cloudman melanoma and human breast carcinoma lines (with mostly type I PKA) are inhibited by cAMP (9). Furthermore, the switch to type I PKA that was recorded in proliferating cancer cell lines was dependent on high, pharmacologically induced levels of cellular cAMP (9). Indeed, most cells respond to high cAMP levels with inhibition of growth but some, such as lymphocytes and melanoma cells, are actually stimulated by low cAMP levels (9). Thus, it is not premature to say that the dysregulated cAMP response of PKA activity in *Prkar1a*$^{-/-}$ and tTA/X2AS cells is at least in part respon-

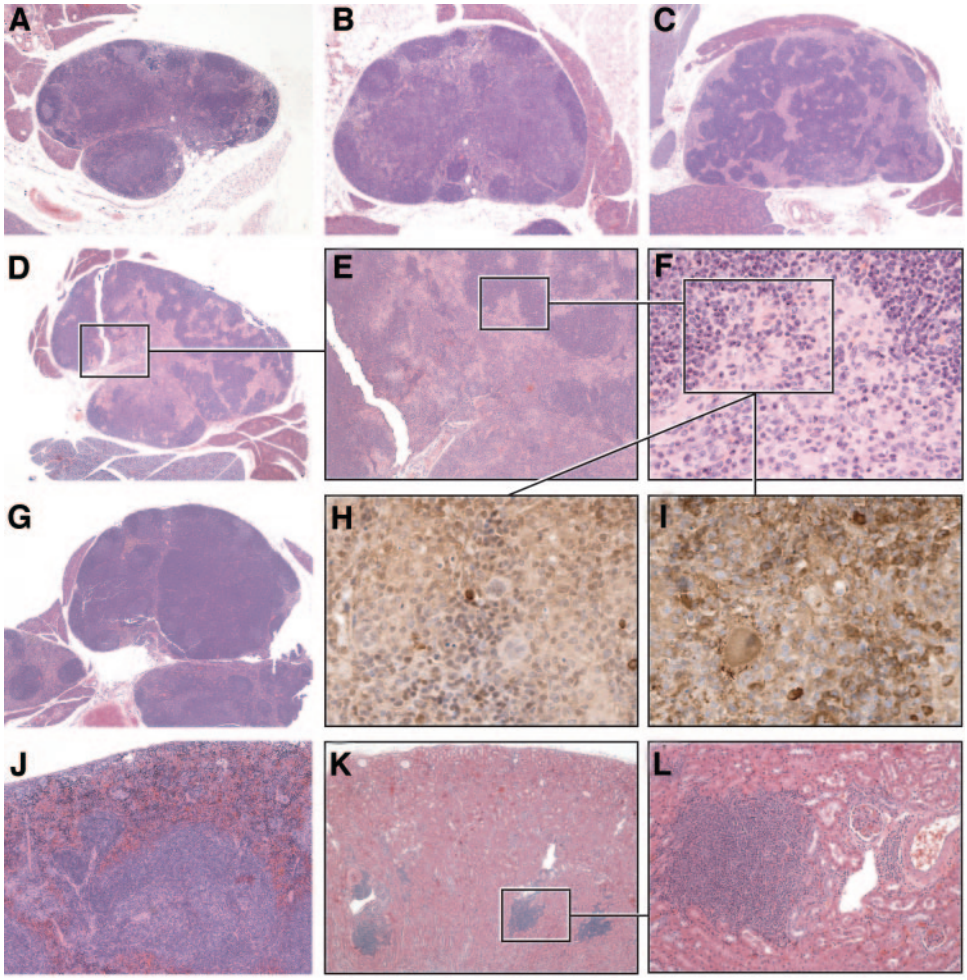

Fig. 4. Histiocytic hyperplasia and lymphomas in mice with down-regulated *Prkar1a*. *A*, normal lymph node from a control mouse (hematoxylin and eosin staining, ×10). Lymph nodes from tTA/X2AS mice with various degrees of histiocytic hyperplasia (*B–F*) and lymphoma (*G*; hematoxylin and eosin staining). In a node with histiocytic hyperplasia, hyperplastic areas show reduction of immunostaining for RIα (*H*) and an increase in RIIβ (*I*; ×40). *J*, spleen from a tTA/Carney complex mouse that died of a metastatic lymphoma (hematoxylin and eosin staining, ×10). *K* and *L*, metastatic lymphoma in the kidney of a tTA/Carney complex mouse (hematoxylin and eosin staining, ×10).

sible for the pathology we observed. Additionally, there may also be some PKA-independent effects of *Prkar1a* that contributed to the phenotype (6). In conclusion, our study suggests that *Prkar1a* down-regulation leads to tumor formation in mice. It is hoped that this mouse model will shed light on some fundamental questions of tumor biology, such as the relationship between cAMP and cellular proliferation, tissue-dependent expression of certain signaling pathways, and the progress from hyperplasia to tumor formation in endocrine and other organs.

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